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THE VARIABILITY OF TWO STRAINS OF STREPTOCOCCUS LACTICUS*

P. G. HEINEMANN

(From the Bacteriological Laboratory, University of Chicago, Chicago)†

Much attention has been given during recent years to methods of classification of streptococci. The literature has been fully discussed by several authors and a review here is unnecessary. Practically all the work reported was carried on by collecting streptococci from different sources and testing their fermentative powers on certain substances by the amounts of acid formed. Hemolysis, reaction in litmus milk, and morphology have also been taken into consideration. Rogers,[1] among others, has pointed out that morphology is of little constancy and depends largely on the kind of available food. Acid formation and hemolytic power have been considered by some to be of sufficient constancy to determine the origin of the culture with some degree of certainty. Whether or not these properties remain constant under changed conditions has not to my knowledge been systematically investigated.

In the work reported in this paper I have approached the subject from a novel viewpoint. Instead of isolating a large number of streptococci, I have selected two strains of the streptococcus lacticus and subjected them to definite conditions. The different tests were repeated at certain intervals during the progress of the work, the object of which was to determine in what manner reactions may be influenced by environment.

The following test substances were used to determine the amount of acid formed: dextrose, lactose, saccharose, raffinose, inulin, salicin, and mannite. One percent of these substances was dissolved in a medium prepared by covering chopped beef with water in the proportion of one liter of water to one pound of beef. After soaking overnight a culture of the bacillus coli was added and the mixture incubated at 37 C. for twenty-four hours. The medium was then heated to 60 C. and held at this temperature for two hours. Then, the temperature was raised to the boiling point and held there for thirty minutes. The meat was pressed out and the remaining meat mixed again with water and pressed a second time. The fluids were mixed

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[1] Jour. Agr. Research, 1914, 1, p. 491.

and the measure brought to the requisite amount, i. e. 1,000 c.c. fluid for each pound of meat. Ten liters were prepared at a time. In the boiling liquid 1 percent Witte peptone and 0.5 percent sodium chlorid were dissolved. After solution, 6 gm. calcium carbonate for each liter were added and the mixture was boiled until the reaction was about 0.3 percent acid to phenolphthalein. This method of neutralization was preferred to the usual one of adding sodium hydrate, since minute amounts of alkali render carbohydrates subject to easy decomposition, while they are relatively stable in an acid reaction. By using calcium carbonate, even the small amount of alkali necessary to produce a pink coloration with phenolphthalein is avoided. It required boiling with calcium carbonate from thirty minutes to an hour to obtain the desired reaction. The medium was then sterilized in large Erlenmeyer flasks without filtration, so that any acid that might form during sterilization would be neutralized by the excess of calcium carbonate. The medium was filtered in a boiling hot condition before the test substances were dissolved. Carbon dioxid was therefore completely removed and no calcium carbonate held in solution. Possible error from decomposed carbohydrates by the presence of alkali was thus avoided.

After solution of the test substances, the medium was distributed in small Erlenmeyer flasks and Nessler tubes. The object was to vary progressively the amount of available free oxygen to the amount of fluid. The largest amount of free oxygen was available in small Erlenmeyer flasks by the large surface offered by 20 c.c. of the fluid. Then, a series of Nessler tubes containing 20, 30, 40, and 50 c.c., respectively, were filled. Finally, another Nessler tube was filled with 50 c.c. and covered with boiled paraffin oil. By this method, each test substance was contained in six lots, each lot offering less free oxygen in proportion until finally anaerobic conditions had been established.

The chief object of the study was to determine whether the original fermentative ability, as indicated by amount of acid formed, would remain the same or be changed by animal passage of the strain. As the streptococcus lacticus is non-pathogenic in its natural condition, large doses are required to produce reactions in animals. The surface of a twenty-four-hour culture on North medium was suspended in physiological salt solution and injected subcutaneously into a rabbit, and the surface of another tube similarly prepared injected into a guinea-pig. The organism was recovered as soon as pronounced lesions appeared, usually in from two to three days. After twenty-four hours' cultivation on North medium, the recovered organism was again inoculated into a rabbit and a guinea-pig. This process was repeated a number of times with the result that virulence was greatly enhanced. Great caution was used when removing material from the lesions for recovery of the organism. The abdomen was shaved, washed in 0.2 percent mercuric chlorid solution and this washed off with alcohol. This procedure was followed before all injections. The material was removed with a platinum loop and streaked on North medium. Streptococci are widely distributed and unless care is exercised there is the possibility that some other strain is obtained instead of the desired one. That this did not happen is proved by cultural tests and chiefly by the fact that the changes in fermentation reactions were distinctly progressive. Suspicious cultures were discarded and another organism was isolated from the same lesion.

The original strains and all strains recovered from animals were cultivated on North medium and transferred after twenty-four-hours' growth to the Erlenmeyer flasks and Nessler tubes prepared in the described manner. Suitable wooden stands were used to hold a series of Nessler tubes. Thus, a strain of the streptococcus lacticus was inoculated into an Erlenmeyer flask containing dextrose dissolved in the medium and into five Nessler tubes containing 20, 30, 40, 50, and 50 c.c., respectively; the last tube containing 50 c.c. was covered with oil after inoculation. The same scheme was carried out with flasks and tubes containing lactose, saccharose, raffinose, inulin, salicin, and mannite. In addition, blank tubes containing each of the test substances dissolved in the medium were prepared. All flasks and tubes were incubated at 37 C. After the lapse of three days the flasks and tubes were shaken and five c.c. removed with sterile pipettes. The 5 c.c. were mixed with 45 c.c. distilled water and, after addition of 1 c.c. of a 1 percent solution of phenolphthalein, titrated with 0.05 normal potassium hydrate solution. The acidity of the blank was deducted from the amount obtained in the inoculated tubes.

The organism was also streaked periodically on blood agar. Human blood and goat's blood were tested. Beef infusion agar was put into tubes, each one containing 9.5 c.c. agar. After melting and cooling to 55 C., 5 c.c. of the defibrinated blood were mixed with the agar, the mixture poured on Petri dishes, and incubated for twenty-four hours to eliminate contamination. A suspension of the organism was streaked on the surface of the blood agar with a platinum needle, the end of which was triangular and hammered flat.

Cultures were made from day to day on North medium in litmus milk and in dilute horse serum. This latter was prepared by diluting horse serum with five times its volume of water and sterilizing. After sterilization there was some precipitate in the diluted serum, but it remained liquid, which was the object of dilution. The milk cultures were observed after twenty-four hours, two days, three days, and ten days. Stains from all cultures were made after twenty-four hours. The milk cultures were incubated in duplicate, one series at 37 C. and the other at room temperature.

The routine outlined was carried on with the original cultures and repeated after each animal passage. At the conclusion of the work, the second strain, which had been passed through milk fifty-eight times on consecutive days, was again tested. In addition, a hemolytic strain, obtained through the kindness of Dr. E. C. Rosenow, was carried through some of the tests for comparison. Work with the first strain was completed before work with the second strain was commenced. The first series was not so complete as the second series, since additional tests were designed for the second series. However, the results of the two series are similar, so that one may be considered as confirmation of the results of the other.

The work will be taken up in the following order: isolation of strains of the streptococcus lacticus; morphology and ocular observations in media; virulence, and lesions produced; acid production in the seven test substances; and influence of amount of available oxygen.

ISOLATION OF STRAINS OF STREPTOCOCCUS LACTICUS

Strain 1 was isolated from ice cream. The liquefied cream was plated in several dilutions in dextrose litmus agar and after two days' incubation at 37 C. nine streptococcus-like colonies were transferred to slanted dextrose agar tubes. After twenty-four hours some of the surface growth was plated again. After colonies from the second set of plates were transferred to dextrose agar, stains were prepared. The final selection gave a strain with the following characteristics: Rather small; frequent chains of six to nine members; many diplococci and decidedly round individual cocci. The Gram stain was positive and no capsules could be demonstrated by using Rosenow's capsule stain. Gelatin was not liquefied. Surface growths on agar and on North media were composed of small colonies giving the appearance of a thin veil. Growth on North medium was always more abundant than on dextrose agar. Litmus milk was acidified after twenty-four hours and completely coagulated after forty-eight hours. The

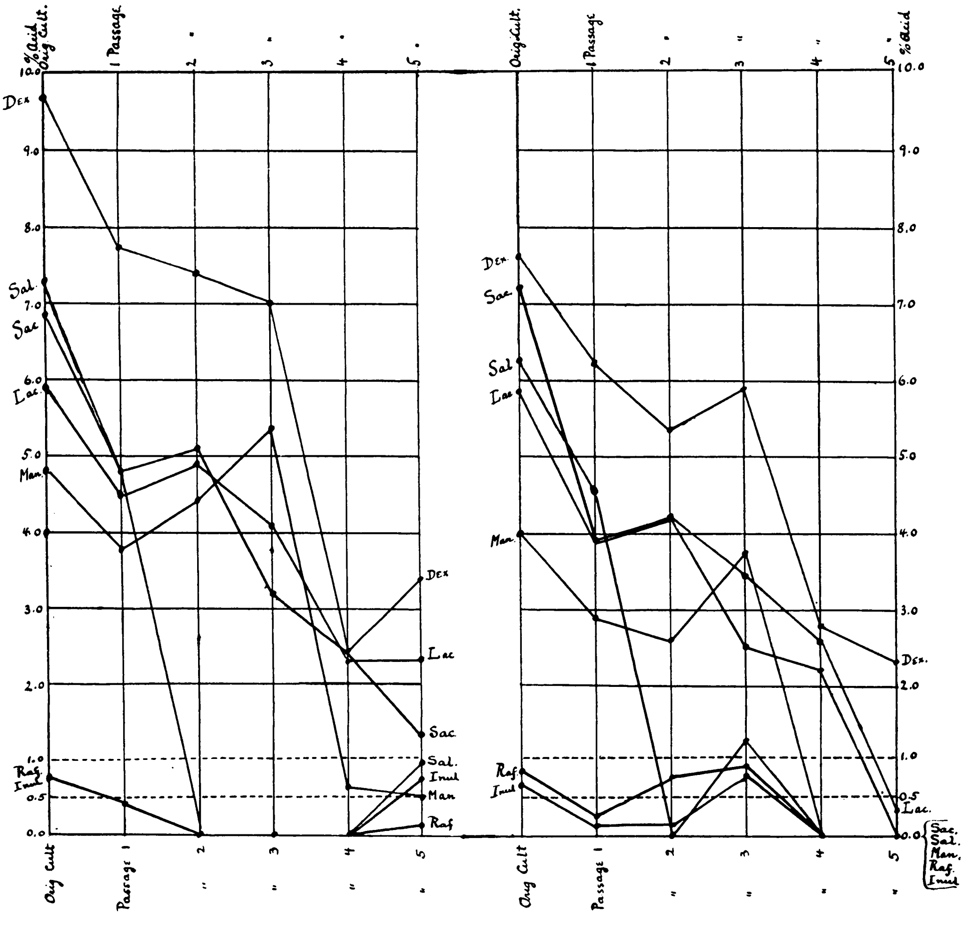

Chart 1.—Results of fermentations of strains obtained by animal passage of Strain 1, expressed in terms of normal acid.

coagulum was decolorized excepting a small pink ring at the surface. A flask containing 500 c.c. milk, sterilized in the autoclave, was inoculated with the contents of a tube of litmus milk containing twenty-four hours' growth. The milk in the flask was coagulated after twenty-four hours. The coagulum was smooth with very little whey. The taste was typical of lactic streptococci, excepting the pronounced cooked taste due to sterilization. This strain did not hemolize either human or goat's blood agar. The culture has now been in the laboratory collection for nearly a year, during which time it was kept active by periodic transfers. At present it forms chains of enormous length, consisting occasionally of sixty to eighty members.

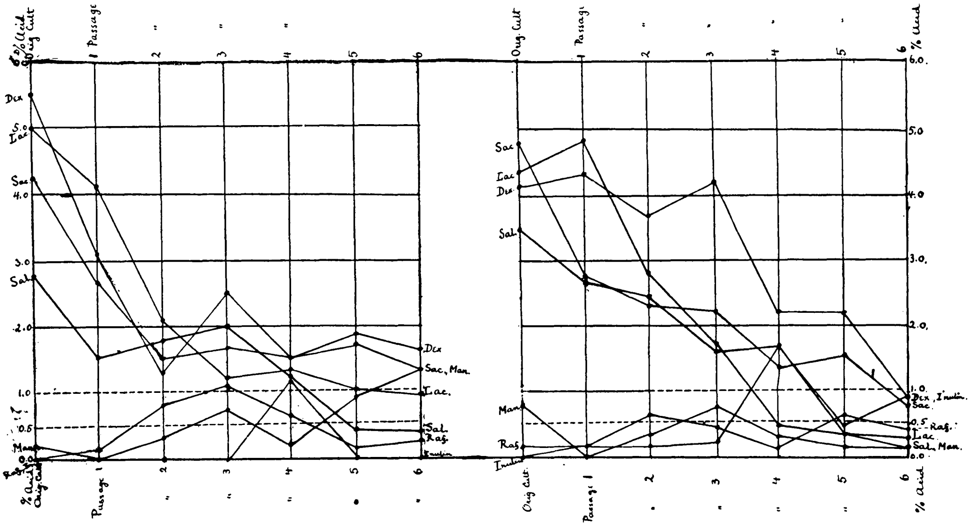

Chart 2.—Results of fermentations of strains obtained by animal passage of Strain 2, expressed in terms of normal acid.

Strain 2 was isolated from milk. Certified milk was incubated at 37 C. for two days. Plates were then prepared from the loppered milk in the same manner as previously described from ice cream. Eleven colonies were transferred to slanted dextrose agar. Of these, six were replated and studied carefully as they did not show distinct morphological differences. They were stained, inoculated on North medium, litmus milk, and gelatin. Surface cultures from North medium were suspended in physiological salt solution and inoculated into a rabbit and a guinea-pig. Streaks were made on human and goat's blood agar. It seemed important to make these tests to avoid

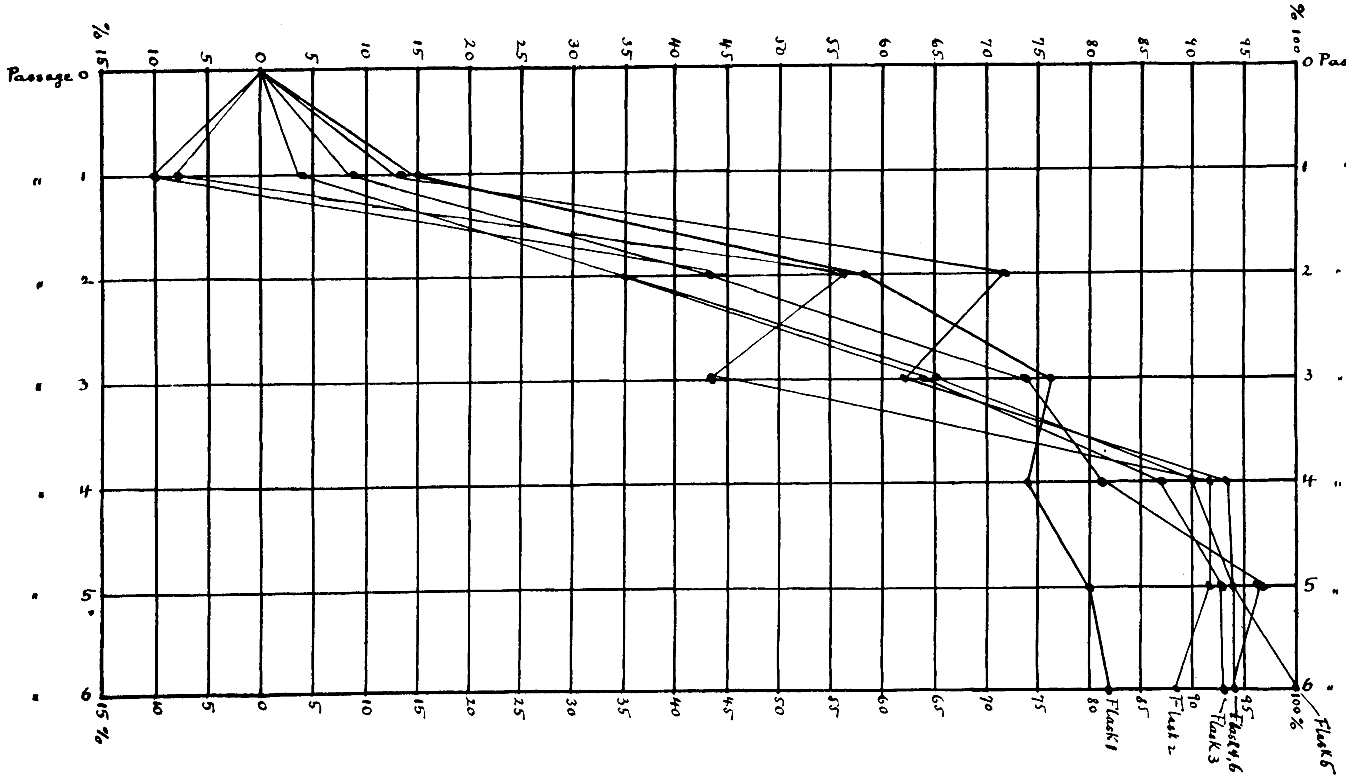

Chart 3.—Percent loss of fermentative power of Strain 2 in lactose. Rabbit passage.

selecting a strain that might be possessed of properties usually taken to indicate pathogenicity. The animals developed no severe lesions. A slight swelling and reddening at the point of injection were all that could be observed. There was no trace of green color or hemolysis on either human or goat's blood agar. One of the strains tested was finally selected. It resembled Strain 1 in all cultural characteristics, excepting that chain formation was less pronounced. Diplococcus forms were prominent and the diplococcus arrangement persisted in the short chains which occasionally were present. It did not liquefy gelatin, was gram-positive, and coagulated milk in forty-eight hours, both at room temperature and at 37 C. The growth on North medium was similar to that of Strain 1. When inoculated into 500 c.c. sterilized milk, the coagulum and taste were similar to those of Strain 1.

The hemolytic strain was passed through the same tests. It acted promptly on animals, causing pus formation; one rabbit died after twenty days and one guinea-pig recovered. Milk was not coagulated in ten days, but acid was produced. Morphologically, it did not differ from the strains of the streptococcus lacticus. The cocci were small and spherical, the chains contained up to ten members, the Gram stain was positive, and no capsules could be demonstrated. When inoculated into 500 c.c. sterilized milk, no coagulum was formed for ten days. No observation was made after this period. Gelatin was not liquefied and surface growth was delicate and veil like. Hemolysis was distinct on human and goat's blood agar.

MORPHOLOGY AND REACTIONS IN CULTURE MEDIA

Strain 1 was passed through litmus milk on thirty-six consecutive days. The morphology did not change, but the time for complete coagulation was reduced to eighteen hours. After the third rabbit passage and the fourth guinea-pig passage, milk was not coagulated in seventy-two hours. The culture was passed through horse serum on six successive days. Tendency to chain formation was more distinct in this medium than in milk. Chains of eight to ten members were more frequent. After the fourth rabbit passage diplococci were scarce, the culture consisting chiefly of chains, altho long chains were not observed. At no time was it possible to demonstrate capsules.

Observations on hemolysis were always made after twenty-four hours' incubation at 37 C. No hemolysis was observed after thirty-six transfers through milk. Horse serum cultures were non-hemolytic. Animal passage gradually developed this property. After the third guinea-pig passage there was slight hemolysis. It was distinct in the thinner parts of the plate but not clear in the thicker parts. After the fourth guinea-pig passage, hemolysis was distinct in all parts of the plate. The clear ring was small. Hemolysis was distinct after the third rabbit passage. Two of the plates were contaminated with a few colonies of the bacillus subtilis. Whenever this occurred the colonies near the bacillus subtilis colonies had a greenish tinge. This phenomenon has been reported by Rosenow.[2]

More extensive studies were made with Strain 2. The original culture coagulated milk completely in somewhat less than forty-eight hours. After thirteen transfers through milk, coagulation was complete in twenty-four hours at 37 C. and after eighteen hours at room temperature. Chains appeared in all milk cultures, but diplococci were most numerous. The chains were chiefly composed of diplococci, and were frequently seen with eight to ten members. Chain formation in the room-temperature cultures seemed to be more common than at 37 C. After thirty-three transfers through milk, chains of twelve to fourteen pairs were frequently observed. Capsules were not demonstrated at any time.

A culture obtained through the kindness of Professor E. G. Hastings was compared with Strain 2. This culture was found in almost pure culture in the milk from a cow without udder trouble. The cow was kept under observation by Professor Hastings, but no udder lesions developed subsequently. This streptococcus coagulated milk in twenty-four hours and agreed morphologically in every respect with Strain 2. The lenght of chains also was about the same. This organism was non-pathogenic to rabbits and guinea-pigs. No attempt was made to produce virulence of this strain by animal passage.

Strain 2 and the hemolytic strain were passed through horse serum ten times. Chains of considerable length and composed of diplococci were predominant after two transfers.

Milk cultures from the second rabbit passage did not coagulate milk in seventy-two hours either at 37 C. or at room temperature. After animal passage, strains frequently showed numerous diplococci of peculiar shape. The individual cocci seemed to be elongated at right angles to the chain axis, thus resembling the so-called picket-fence ("stakett") form. Guinea-pig passage did not seem to affect the power of coagulating milk as much as rabbit passage. Even after the sixth guinea-pig passage, milk was completely coagulated after forty-eight hours. Decolorization of the litmus, however, was not complete as it was in earlier cultures.

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[2] Jour. Infect. Dis., 1914, 14, p. 1.

The above-noted picket-fence diplococci became more numerous with the number of passages. Cultures from the third rabbit passage occasionally showed several of these diplococci joined to a chain.

Cultures from the second rabbit passage hemolized both human and goat's blood agar. The clear circles of hemolysis were small. The hemolytic power persisted through cultures from all subsequent passages. Cultures from guinea-pig passages showed no hemolysis before the fifth passage.

VIRULENCE AND LESIONS PRODUCED

Guinea-pig Passages.—The first injection of Strain 1 produced a small, indurated area. On opening after three days, a small amount of blood and serum was discharged which gave a pure culture of streptococci. The second injection caused necrosis of tissue and the formation of a small pocket of pus. A smear from the pus showed streptococci in pure culture. The amount of pus increased with succeeding passages, but no generalized infection took place, and the animals eventually recovered.

A guinea-pig was injected with 5 c.c. of a seventh transfer of Strain 1 in horse serum. The animal died after eleven days. The peritoneal and pleural cavities were filled with a bloody fluid; the lungs were normal; the liver filled with small pus centers; and the spleen was very dark. Streptococci were recovered from the heart's blood, the peritoneal fluid, and the spleen.

The first injection of Strain 2 produced a small, indurated area, somewhat swollen and reddened. The organism was recovered after three days and injected into Guinea-pig 2. A small pus pocket formed. In the third guinea-pig passage, the indurated area was large with considerable pus. The symptoms increased until in the fifth animal an area covering a large part of the abdomen was indurated and swollen. A large pus pocket had formed and the animal died after twenty-two days. No autopsy was made as post mortem infection had taken place before the death of the animal was discovered. The sixth guinea-pig died after nineteen days. There was much pus over the abdomen, and the inguinal glands were enlarged. Streptococci were recovered from the pus and the heart's blood. The seventh guinea-pig died after twelve days. No lesions were found in internal organs. On the abdomen a hard lump was found and much pus of tough consistency. Streptococci were recovered from the pus, the heart's blood, and the peritoneal fluid. The first four guinea-pigs recovered.

A culture of the original strain was injected into the peritoneal cavity of a guinea-pig without any apparent effect.

Rabbit Passages.—Virulence was acquired in shorter time and in greater measure by rabbit than by guinea-pig passage. The first injection of Strain 1 produced a soft, reddish area. On opening a serous fluid was discharged, which gave a pure culture of streptococci. The second injection produced a much larger area with a small center of pus. The third injection produced a large, inflamed area filled with pus which contained streptococci in pure culture. The rabbit had considerable temperature. The fourth injection produced similar, but more severe, symptoms. This rabbit died after fifteen days. The inguinal glands were swollen and the peritoneal and pleural cavities contained pus. Streptococci were recovered from the pus, the heart's blood, the liver, and the kidney. The fifth injection produced a large amount of pus and the animal became emaciated, but recovered.

The first injection of Strain 2 produced a small hyperemic area. There was a small amount of serum, which contained streptococci in pure culture. The second injection produced a small amount of pus. The third and fourth injections produced inflamed areas and pus in larger quantities. The fifth injection was followed by the formation of an enormous swelling containing some pus and a large amount of serum. This rabbit died after twenty-one days. The inguinal glands were enlarged, the kidneys congested, and pus foci were found in the lungs. Streptococci were recovered from the heart's blood and the pus in the lungs. After the sixth injection a large swelling developed which was filled with pus. The rabbit died in fifteen days. The inguinal glands were extraordinarily large. The other lesions were similar to the fifth rabbit. Streptococci were recovered from the pus, lungs, heart's blood, kidneys, and inguinal glands.

The hemolytic strain was injected subcutaneously into a rabbit. The animal died in twelve days. The inguinal glands were swollen and inflamed, pus was found at the point of injection, in the lungs, and peritoneum, and the kidneys, liver and spleen were congested. The organism was recovered from all organs and the heart's blood.

TABLE 1

AMOUNT OF ACID PRODUCED BY STREPTOCOCCUS LACTICUS (STRAIN 1)

Substance	Flask	Original Culture	Rabbit Passage					Orig. inal Culture	Guinea-pig Passage			
			1	2	3	4	5		1	2	3	4
Dextrose........	1	9.7	7.8	7.4	7.0	2.4	3.2	9.7	6.8	6.8	6.2	2.6
	2	8.1	6.1	6.1	6.1	2.0	2.3	8.1	6.1	6.4	6.5	3.7
	3	7.3	6.9	5.9	5.9	2.3	2.1	7.3	6.1	6.3	6.8	2.1
	4	7.5	6.4	5.9	5.9	2.6	2.5	7.5	6.2	6.3	6.5	2.0
	5	7.5	6.9	5.8	5.9	2.8	2.3	7.5	6.1	6.4	6.5	1.3
	6	7.6	6.2	5.3	5.9	2.8	2.3	7.6	5.9	6.0	6.6	2.5
Lactose.........	1	6.0	4.5	4.9	4.1	2.3	2.3	6.0	4.1	4.2	4.9	1.5
	2	6.1	4.1	4.7	3.4	2.6	0.0	6.1	3.7	3.8	3.9	0.3
	3	6.1	4.2	4.5	3.4	2.7	0.1	6.1	3.8	3.8	4.1	0.6
	4	5.9	4.0	4.3	3.2	2.9	0.2	5.9	3.9	3.9	4.1	0.0
	5	5.7	4.1	4.1	3.1	2.8	0.5	5.7	4.3	3.8	4.2	0.0
	6	5.9	3.9	4.2	3.4	2.6	0.3	5.9	3.3	3.9	4.1	0.9
Saccharose......	1	6.9	4.8	5.1	3.2	2.4	1.3	6.9	5.5	5.1	5.5	1.1
	2	6.7	4.1	3.9	2.3	2.3	0.4	6.7	4.5	5.1	5.0	1.1
	3	6.9	4.0	4.9	2.3	2.2	0.1	6.9	4.4	4.7	5.2	0.0
	4	6.8	4.3	4.7	3.1	2.0	0.2	6.8	4.5	5.3	5.2	0.0
	5	7.1	4.1	4.6	1.9	2.1	0.3	7.1	4.5	5.3	5.4	0.0
	6	7.2	3.9	4.2	2.5	2.2	0.1	7.2	4.6	5.3	5.4	0.9
Raffinose.......	1	0.7	0.4	0.0	0.0	0.0	0.1	0.7	0.4	4.7	1.1	0.7
	2	0.9	0.3	0.2	0.5	0.9	0.0	0.9	0.2	2.5	0.0	0.0
	3	0.8	0.3	0.5	0.6	0.0	0.0	0.8	0.2	2.5	0.0	0.0
	4	0.9	0.3	0.6	0.5	0.0	0.0	0.9	0.2	3.7	0.0	0.0
	5	0.9	0.3	0.3	0.7	0.0	0.0	0.9	0.2	2.7	0.0	0.0
	6	0.8	0.2	0.7	0.9	0.0	0.0	0.8	0.3	7.7	0.0	0.0
Inulin..........	1	0.7	0.4	0.4	0.2	0.0	0.7	0.7	0.4	1.5	0.0	0.6
	2	0.6	0.1	0.0	2.0	0.0	0.0	0.6	0.3	2.4	0.0	0.0
	3	0.6	0.2	0.1	0.7	0.0	0.0	0.6	0.2	2.4	0.0	0.0
	4	0.7	0.2	0.1	0.8	0.0	0.0	0.7	0.2	2.9	0.0	0.0
	5	0.8	0.2	0.2	0.8	0.0	0.0	0.8	0.2	2.7	0.0	0.0
	6	0.6	0.1	0.1	0.8	0.0	0.0	0.6	0.2	3.1	0.3	0.0
Salicin..........	1	7.3	4.8	0.0	0.0	0.0	0.9	7.3	5.7	0.1	6.3	0.5
	2	6.1	4.7	0.0	1.1	0.0	0.0	6.1	4.7	0.7	6.0	0.3
	3	6.1	5.4	0.0	1.1	0.0	0.0	6.1	4.6	1.3	6.0	0.0
	4	5.9	4.7	0.0	1.1	0.0	0.0	5.9	4.8	0.6	5.8	0.0
	5	6.3	4.7	0.0	1.2	0.0	0.0	6.3	4.7	1.2	5.8	0.0
	6	6.3	4.5	0.0	1.2	0.0	0.0	6.3	4.6	1.5	5.5	0.0
Mannite........	1	4.8	3.8	4.4	5.3	0.6	0.5	4.8	3.9	4.6	4.9	0.7
	2	3.7	3.7	2.9	2.7	0.0	0.0	3.7	2.8	3.6	3.4	0.0
	3	3.8	2.9	3.0	3.3	0.0	0.0	3.8	2.8	3.6	3.3	0.0
	4	3.6	3.6	2.7	2.6	0.0	0.0	3.6	2.5	2.7	3.4	0.0
	5	3.7	2.9	2.9	2.5	0.0	0.0	3.7	2.4	2.7	3.4	0.0
	6	4.0	2.9	2.6	3.7	0.0	0.0	4.0	2.9	3.2	2.8	0.0

TABLE 2

AMOUNT OF ACID PRODUCED BY STREPTOCOCCUS LACTICUS (STRAIN 2)

Substance	Flask	Original Culture	Rabbit Passage						Original ture Cul-	Guinea-pig Passage						
			1	2	3	4	5	6		1	2	3	4	5	6	7
Dextrose....	1	5.5	3.1	1.3	2.5	1.5	1.9	2.6	5.5	3.9	3.9	3.3	3.1	2.7	2.3	1.6
	2	5.2	4.0	3.6	3.0	3.2	1.9	1.4	5.2	5.4	4.9	4.6	3.1	3.0	2.8	1.6
	3	5.1	4.3	3.1	2.7	1.6	1.7	1.2	5.1	5.0	5.1	4.1	3.0	2.9	3.0	1.5
	4	5.0	4.1	3.0	1.5	1.1	1.4	1.0	5.0	4.9	5.1	3.8	3.1	2.2	3.0	1.3
	5	5.1	4.0	3.1	1.7	1.1	1.4	0.9	5.1	4.9	5.4	3.7	3.4	3.2	2.4	2.9
	6	4.1	4.3	3.7	4.2	2.2	2.2	0.9	4.1	4.4	4.7	4.0	1.1	3.2	2.9	2.2
Lactose.....	1	5.0	4.1	2.1	1.2	1.3	1.0	0.9	5.0	5.2	5.1	3.8	2.5	2.9	1.8	0.9
	2	4.9	5.5	2.1	2.8	0.4	0.4	0.6	4.9	5.1	4.7	4.1	1.6	2.9	2.9	0.9
	3	4.6	4.4	3.0	1.7	0.6	0.3	0.3	4.6	5.1	4.7	4.1	3.1	3.1	1.4	0.9
	4	5.3	4.8	3.0	1.4	1.0	0.2	0.3	5.3	5.0	4.8	3.7	3.1	3.1	1.7	1.1
	5	5.1	4.3	1.5	2.0	0.4	0.3	0.0	5.1	5.1	4.8	4.4	3.1	2.9	1.6	0.1
	6	4.3	4.8	2.8	1.7	0.5	0.3	0.3	4.3	5.1	5.5	4.5	2.9	1.7	3.0	0.8
Saccharose.	1	4.2	2.7	1.5	1.7	1.3	1.7	1.3	4.2	2.5	2.7	2.8	2.4	2.2	2.9	2.2
	2	4.6	3.6	3.1	3.2	1.4	1.6	1.3	4.6	4.6	4.7	2.1	2.7	2.8	2.6	2.1
	3	4.7	3.2	3.2	1.8	1.4	1.5	1.4	4.7	4.5	4.9	2.1	2.9	1.0	1.9	2.0
	4	4.6	3.1	3.2	3.3	1.1	1.4	1.2	4.6	4.7	4.9	2.2	2.8	2.7	2.8	1.6
	5	4.8	3.5	3.3	2.8	1.1	1.4	1.3	4.8	4.8	4.9	3.0	3.1	2.9	1.8	1.6
	6	4.8	2.8	2.3	2.2	1.3	1.5	0.7	4.8	4.7	4.8	2.9	2.4	1.6	2.9	2.9
Raffinose...	1	0.0	0.1	0.8	1.1	0.6	0.1	0.2	0.0	0.0	0.0	0.5	1.8	1.1	0.7	0.0
	2	0.1	0.1	0.7	0.2	0.3	0.6	0.3	0.1	0.1	0.1	0.0	1.6	2.0	1.1	0.9
	3	0.1	0.0	0.6	0.3	0.3	0.1	0.3	0.1	0.0	0.0	0.0	0.9	1.2	0.9	0.2
	4	0.0	0.0	0.6	0.4	0.1	0.0	0.3	0.0	0.0	0.0	0.5	1.0	1.7	1.8	0.7
	5	0.0	0.1	0.8	0.3	0.2	0.1	0.2	0.0	0.0	0.6	0.0	1.7	1.8	0.9	0.3
	6	0.1	0.1	0.6	0.4	0.1	0.6	0.4	0.1	0.3	0.6	0.8	1.6	1.9	0.9	0.3
Inulin.......	1	0.0	0.1	0.0	0.0	1.2	0.0	0.0	0.0	0.5	0.9	0.1	1.2	1.1	0.0	0.9
	2	0.0	0.1	0.6	0.2	0.6	0.2	0.8	0.0	0.2	0.0	1.2	1.0	0.9	0.0	0.2
	3	0.0	0.0	0.6	0.3	0.7	0.3	1.0	0.0	0.1	0.0	1.2	1.1	0.8	0.3	0.3
	4	0.0	0.0	0.6	0.2	0.6	0.1	0.7	0.0	0.0	0.0	0.0	1.2	0.8	0.6	0.3
	5	0.0	0.2	0.1	0.2	0.6	0.2	0.9	0.0	0.1	0.0	0.1	1.2	0.8	0.1	0.2
	6	0.0	0.1	0.1	0.2	1.7	0.4	0.9	0.0	0.2	0.0	0.0	1.4	1.0	0.6	0.2
Salicin......	1	2.8	1.5	1.8	2.0	1.2	0.4	0.3	2.8	2.4	2.9	2.4	2.5	1.6	1.6	1.9
	2	3.1	2.5	2.2	1.8	0.6	0.4	0.2	3.1	3.2	3.5	2.2	1.8	2.1	2.2	2.1
	3	3.7	2.6	2.0	1.7	0.7	0.2	0.3	3.7	3.4	3.2	2.2	2.2	1.8	1.9	2.1
	4	3.4	2.9	1.6	1.9	0.6	0.3	0.1	3.4	3.2	3.5	2.3	2.1	1.9	2.1	1.9
	5	3.7	2.6	1.5	1.9	0.6	0.2	0.1	3.7	3.1	3.2	2.3	2.4	2.1	2.3	2.1
	6	3.5	2.7	2.4	1.6	1.7	0.3	0.1	3.5	3.3	3.2	2.3	2.3	2.2	2.2	1.8
Mannite....	1	0.2	0.0	0.3	0.7	0.2	0.9	1.3	0.2	0.1	0.9	0.0	1.9	1.2	1.6	0.3
	2	0.1	0.0	0.4	0.2	0.6	0.2	0.3	0.1	0.2	0.8	0.0	1.8	1.1	1.1	0.1
	3	0.3	0.0	0.3	0.3	0.4	0.2	0.3	0.3	0.0	0.8	0.0	1.6	0.9	1.1	0.2
	4	0.1	0.0	0.2	0.2	0.3	0.2	0.2	0.1	0.0	0.2	0.0	1.3	1.0	1.1	0.0
	5	0.2	0.0	0.3	0.2	0.2	0.0	0.1	0.2	0.1	0.1	0.0	1.4	1.0	1.2	0.3
	6	0.7	0.0	0.3	0.7	0.3	0.1	0.1	0.7	0.1	0.0	0.3	1.2	0.9	1.0	0.3

FERMENTATION REACTIONS

Both strains were inoculated into broth containing 1 percent of the test substances. This was repeated after each animal passage through rabbits and guinea-pigs and a control test of Strain 2 made of the milk culture after fifty-eight transfers through litmus milk. This was done in accordance with the technic outlined. In Tables 1 and 2, which show the quantities of acid formed, the figures represent the number of cubic centimeters of normal alkali required to neutralize the solution after the amount of acid found in the blank has been deducted.

TABLE 3

AMOUNT OF ACID PRODUCED BY STREPTOCOCCUS LACTICUS (STRAIN 2) AFTER FIFTY-EIGHT TRANSFERS THROUGH LITMUS MILK COMPARED WITH THE AMOUNT OF ACID PRODUCED BY THE ORIGINAL STRAIN

Flask	Dextrose		Lactose		Saccharose		Raffinose		Inulin		Salicin		Mannite	
	Passage	Original	Passage	Original	Passage	Orig. inal	Passage	Original	Passage	Orig. inal	Passage	Original	Passage	Original
1..............	3.5	5.5	3.3	5.0	3.2	4.2	0.1	0.0	0.1	0.0	2.6	2.8	0.3	0.2
2..............	4.9	5.2	4.8	4.9	3.2	4.6	0.1	0.1	0.1	0.0	3.4	3.1	0.0	0.1
3..............	4.9	5.1	4.9	4.6	4.6	4.7	0.1	0.1	0.1	0.0	3.2	3.7	0.2	0.3
4..............	4.8	5.0	4.7	5.3	4.0	4.6	0.1	0.0	0.1	0.0	3.2	3.4	0.1	0.1
5..............	4.9	5.1	4.9	5.1	4.2	4.8	0.1	0.0	0.1	0.0	3.4	3.7	0.2	0.2
6..............	4.9	1.1	4.9	4.3	4.3	4.8	0.1	0.1	0.1	0.0	3.3	3.5	0.2	0.7

In the preceding tables, the column "flask" refers to the different exposures to free oxygen. Flask 1 is the Erlenmeyer flask containing 20 c.c. of the medium and offering a large surface to the air; Flask 2 is the Nessler tube with 20 c.c.; Flask 3, the Nessler tube with 30 c.c.; Flask 4, the Nessler tube with 40 c.c.; Flask 5 the Nessler tube with 50 c.c.; and Flask 6, the Nessler tube with 50 c.c. covered with oil for anaerobic cultivation.

For convenience, the results of Flasks 1 and 6 of each series are plotted in Charts 1 and 2. The figures at the top represent the number of the passage and the figures on the side the number of cubic centimeters of normal acid required for neutralization. Dotted lines appear at 1 percent and at 0.5 percent. Winslow[3] takes 0.5 percent as the dividing line between acid formers and non-acid formers. Rogers[4] takes 1 percent as the dividing line, while Hopkins and Lang[5] take 0.8 percent. Whichever amount is taken as indicative of acid production has bearing on the interpretation of the result, so that under one scheme a certain substance may be considered as having been fermented, while under another scheme the same substance would be considered as not having been fermented.

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[3] Jour. Infect. Dis., 1910, 7, p. 1.
[4] Ibid.
[5] Ibid., 1914, 15, p. 63.

I have also figured the percentage of loss of fermentative ability by taking the amount of acid formed originally in the test solution as 0 and then plotting the percentages of loss in ascending curves. Two sets of charts were prepared according to these figures, one set being arranged according to flasks and the other according to the test-substance. Of the thirty charts prepared, Chart 3 is representative, showing the loss of lactose.

DISCUSSION OF RESULTS

A study of morphology leads to the assumption of three facts. Chain formation is favored by cultivation in media containing blood-serum in the absence of carbohydrates. Chain formation is favored also by animal passage. It is not clear from these studies what conditions are required for the production of picket-fence-shaped streptococci. Whether this is due to some particular substance in the medium or whether only certain strains are able to assume this form is not indicated. Perhaps both conditions are essential. Hemolytic power may be developed by animal passage. The ability to coagulate milk increases with frequent transplants through milk and decreases with increasing virulence. As will be shown later, coincident with increasing virulence is decrease of ability to produce acid from carbohydrates. It may be assumed therefore that the decreasing ability to ferment lactose reaches a stage when not sufficient acid is produced to coagulate the milk. The ability to coagulate milk decreased more rapidly by rabbit passage than by guinea-pig passage, while virulence increased more rapidly by rabbit passage than by guinea-pig passage.

The increase of virulence by animal passage has been discussed by me in an earlier paper.[6] The results obtained in this work are in agreement with previously reported work and need not be discussed again.

The fermentation reactions are of particular interest. Several papers have been published recently dealing with the variability in fermentation reactions of several species of bacteria. Goodman[7] succeeded in producing a high acid and a low acid form of diphtheria bacilli from one strain. Winslow and Walker[8] found acid formation by the bacillus paratyphosus relatively stable. These authors made no attempt at variation of environment. Buchanan and Truax[9] tested inheritance of acid production by the streptococcus lacticus. They conclude that "impressed variations do not appear to be inheritable." Rettger and Sherrick[10] conclude from a study of several organisms that "bacterial variation, at least of the fluctuating type, may be brought about by what may be termed artificial selection." These authors suggest that by animal passage avirulent individuals may be eliminated and that by repeated passage elimination takes place repeatedly with production of a pure pathogenic type.

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[6] Ibid., 1907, 4, p. 87.
[7] Ibid., 1908, 5, p. 421.

The changed fermentation reactions resulting from animal passage of the streptococcus lacticus in my experiments may not be constant. Possibly by recultivating the strains, the fermentative power of which was reduced, the original acid production might be re-established. Rettger's theory of selection by animal passage would receive support if the acquired ability should prove to remain constant. This problem awaits further experimentation.

Study of Chart 1, which gives the results of fermentations of strains obtained by animal passage of Strain 1, shows that the fermentative ability of the organism on the test substances is gradually reduced. Salicin is not fermented after the third passage through rabbits and not after the fourth passage through guinea-pigs. After the fifth passage, dextrose, lactose, and saccharose are still fermented after passage through either animal, but the quantity of acid produced is reduced. Mannite is not fermented after the fourth passage. If 0.5 percent acid is taken as dividing line we find that the small amount of acid formed from raffinose and inulin is lost after the first passage, but is regained after the fifth passage. In Flask 2, after the fifth rabbit passage, dextrose only is fermented, while after the fourth guinea-pig passage dextrose and saccharose are fermented. The amount of acid produced from saccharose is so small, however, that probably if a fifth passage had been made it would have been lost entirely.

It does not seem profitable to mention singly the results obtained in all the flasks by both strains. In a general way it may be stated that fermentative ability is gradually reduced by animal passage and that in due time some of the substances yield less than 0.5 percent acid and must be considered non-fermentable. In tubes with reduced oxygen supply, the decrease is more rapid than with free access of oxygen. Thus, the smallest loss is found in Flasks 1 and 2, while the following flasks show a relatively larger percentage of loss. However, the number of the experiments was not large enough to assign a definite ratio for the flasks. The relative decrease of amount of acid formed by rabbit passage is smallest in dextrose, next in saccharose, then follow lactose and salicin. By guinea-pig passage the relative loss in acid formation is smallest in dextrose, followed by saccharose, salicin, and lactose in the order named.

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[8] Ibid., 1909, 6, p. 90.
[9] Ibid., 1910, 7, p. 680.
[10] Jour. Med. Research, 1911, 24, p. 265.

Whether the loss of acid production is due to actual loss of fermentative ability or to reduced growth may be questioned. This possibility was borne in mind during the progress of the work. No actual counts of bacteria were made from each flask, but the turbidity, which appeared after shaking, seemed uniformly similar to the naked eye. In this connection, it was noted that as the chains became more pronounced after animal passages the sediment became heavier and the supernatant fluid clearer.

A few words may be said in reference to the more complex substances, raffinose, salicin, and mannite. After the second rabbit passage salicin was not fermented; after the third, a small amount of acid was formed; and after the fourth and fifth passages no acid was formed. This holds good for Strain 1. For Strain 2 the curves are similar, but the actual differences much smaller. Raffinose and inulin have interesting curves. After the third rabbit passage of Strain 1 and the second guinea-pig passage, both these substances are fermented, but they are not fermented after the fourth rabbit passage and the third guinea-pig passage. I have no explanation for why these more complex substances should be fermented with moderate oxygen supply, but not with free access or total exclusion of oxygen. Mannite was fermented by the original culture of Strain 1, but not by Strain 2. In the fourth and fifth guinea-pig passages, however, there are small amounts of acid formed by Strain 2.

It may be stated in a general way that the acid-producing power of the two strains of the streptococcus lacticus is reduced as the number of animal passages increases. This decrease is rapidly pushed to a point where some of the substances, notably salicin and mannite, are practically not fermented. As a rule, dextrose and saccharose are fermented more persistently than lactose. The figures suggest that more complex substances (raffinose and inulin) may be fermented after animal passage when the original did not ferment them. These two substances are fermented chiefly when the supply of free oxygen is reduced, but not eliminated. The reason is not clear and extensive experiments will have to be made to determine the exact nature of this phenomenon.

If we do not consider smaller amounts of acid than 1 percent, Strain 1 of the streptococcus lacticus before and after animal passages may be classified as fermenting dextrose, lactose, saccharose, salicin, and mannite; or dextrose, lactose, saccharose, and mannite; or dextrose, lactose, and saccharose; or dextrose and saccharose; or finally dextrose only. On the other hand, it may be classified as fermenting also raffinose and inulin.

Strain 2 may be classified as fermenting dextrose, lactose, saccharose, and salicin; or dextrose, lactose, saccharose, raffinose, and salicin; or dextrose, lactose, saccharose, and mannite; or dextrose, lactose, and inulin; or saccharose only; or finally as fermenting none of these seven substances. This consideration emphasizes that the ability to produce acid from test substances can be indicative only of the immediate source of the strain, but cannot serve as a permanent method of classifying streptococci into classes, species, or varieties.

Fermentation results under conditions of the experiments are changed materially and progressively. If we are able to determine the immediate source of a strain of streptococcus by testing its power to produce acid from certain test substances, it becomes necessary to find, if possible, whether conditions have prevailed which may have increased or decreased fermentative ability. In a general way, it may be assumed that streptococci that have been in contact with animal lesions, no matter whether these lesions were caused by the streptococci themselves or by other means, have low fermentative ability. Streptococci that have lived on food rich in carbohydrate show high fermentative ability. We can readily understand how an accidental injury to the udder of a cow, for instance, may favor multiplication of the streptococcus lacticus, which is ordinarily found in milk, and finally production of a variety which is pathogenic, of low fermentative ability, and hemolytic. We may assume that milk usually contains streptococci of different properties and that some of them may be more easily transformed into pathogenic varieties than others. The two strains subjected to animal passage showed originally all characteristics of typical streptococcus lacticus. One, however, fermented mannite, the other did not. Pathogenicity developed in similar ratio in both strains. The fact that Strain 1 retained its spherical shape throughout the experiments while Strain 2 assumed in part the picket-fence form is suggestive. The appearance of picket-fence streptococci accompanied by large numbers of leukocytes in milk, is taken by some sanitarians as indicating mastitis in cows.

The experiments show clearly that great variation is possible in the acid-producing power of the two strains of the streptococcus lacticus. The variability depends on at least two factors: The amount of free available oxygen; the influence of certain nutritive substances. If the organism becomes accustomed to albuminous food in the living animal the power to ferment test substances is reduced. How rapidly the original ability to ferment may be re-established and under what conditions raffinose and inulin may be fermented by strains which originally do not ferment them is an open question. The fermentative ability of the original strains was not changed materially after thirty-eight transfers, respectively, in litmus milk.

CONCLUSIONS

Chain formation of the streptococcus lacticus is favored by animal passage and by cultivation in media containing blood serum, without the addition of carbohydrate.

The power to hemolyze human and goat's blood may be acquired to some extent by animal passage.

The presence of a capsule could not be demonstrated at any time. A pathogenic streptococcus does not necessarily possess a capsule.

Animal passage develops and increases virulence of the streptococcus lacticus, and the virulence develops at a more rapid rate in rabbits than in guinea-pigs.

Acid production in certain test substances is variable. While the amount of acid produced was not materially changed by fifty-eight consecutive transfers through litmus milk, it was materially changed by animal passage.

By animal passage, the amount of acid produced by the original strain progressively decreased, and fermentation of some of the substances was inhibited. Raffinose and inulin, which were not fermented by the original cultures, were attacked with production of relatively small amounts of acid under certain conditions. Moderate oxygen supply seems to favor fermentation of raffinose and inulin. One strain temporarily acquired the ability to ferment mannite.

Presence of free oxygen seems to favor the production of acid. Under anaerobic conditions less acid in proportion was formed than under conditions with free access of oxygen, and under anaerobic conditions a smaller number of test substances was fermented than under aerobic conditions.

The fermentative ability is not changed materially by repeated transfers through litmus milk.